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MECHANISMS OF OXYGEN TOXICITY AT THE CELLULAR LEVEL,(U)
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MECHANISMS OF OXYGEN TOXICITY AT THE CELLULAR LEVEL

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STATUS REPORT, COVERING THE PERIOD SINCE THE ANNUAL REPORT OF JANUARY 1978, AND REQUEST FOR CONTINUATION

This report consists of the following: (I) Summary of Accomplishments, (II) Reseach Remaining to Be Completed this Contract Year, (III) Outline of Future Plans, (IV) Itemized Budget, and Administrative Approval (p. 1).

I. Summary of Accomplishments: A summary of significant positive findings during the total contract period will be used to introduce the specific accomplishments of the current contract.

I.a. SUMMARY

Oxygen at elevated pressures is toxic for life forms from microbes to man. Growth is inhibited in Escherichia coli and results primarily from poisoning of specific enzymes in the following biosynthetic pathways: branched-chain and aromatic amino acids, NAD-niacin, phosphoribosylpyrophos-

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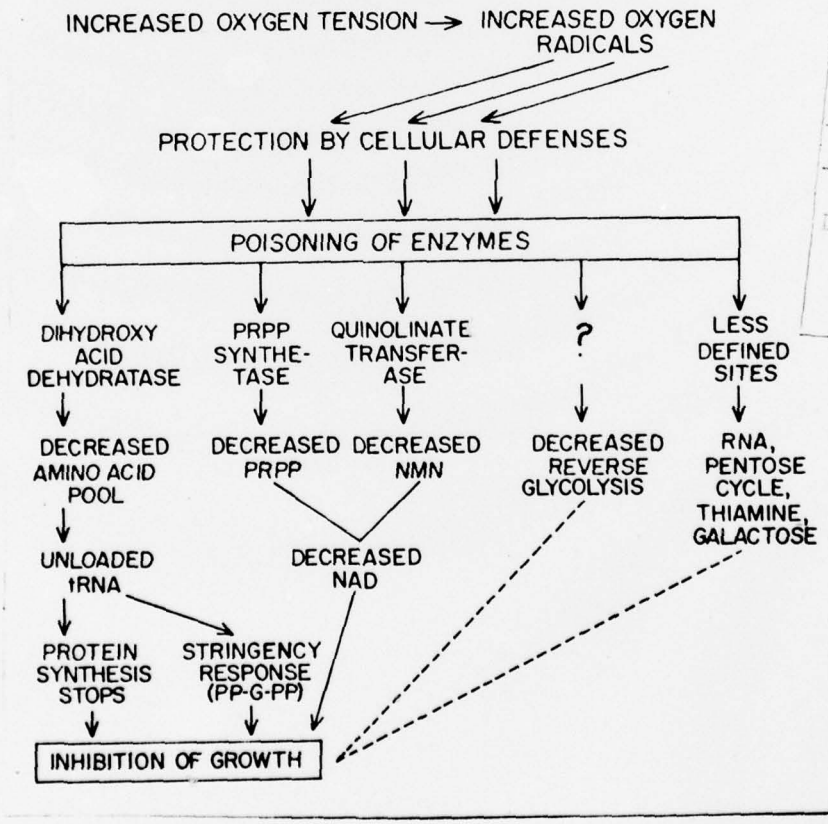
phate (PRPP) and reverse glycolysis. Inhibition of amino acid biosynthesis indirectly stops protein synthesis and induces "stringency" (production of pp-guanine-pp, a powerful inhibitor of metabolic processes) which accounts for inhibition in metabolic processes where there is no observed enzymatic poisoning. Inhibitions in the PRPP, NAD-niacin, and reverse glycolysis pathways are significant to bacterial (and perhaps to human) oxygen toxicity where products of the pathways protect. These relationships are shown in the following diagram (figure 1).

FIGURE 1. BASIC SITES AND INTERRELATED CONSEQUENSES OF OXYGEN POISONING AT THE CELLULAR LEVEL



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I.b. Research completed, as of December 31, 1978, toward specific objectives detailed in the proposal for the period April 1, 1978 - March 31, 1979.

(1) Relationship between guanine polyphosphate (ppGpp) concentration, oxygen inhibition of metaoblism, and the known inhibition of amino acid biosynthesis (part 1-b-i of the proposal).

The ppGpp has been measured in a stringent and in a relaxed strain of E. coli prior to and during oxygen poisoning. Our hypothesis that oxygen poisoning would induce the stringent response, was confirmed. ppGpp increased in concentration in stringent but not in relaxed strains (Figure 2). This supports our previous findings for poisoning of specific enzymes of amino acid biosynthesis which would lead to amino acid starvation and induction of ppGpp production with consequent shut-down of various metaoblic pathways.

We have carried the study further and obtained results beyond those proposed. Specifically, we hypothesized that strigent strains should benefit from the rapid and uniform shut-down of metabolism via rapid ppGpp production during the early exposure to hyperoxia. (By analogy, the stringent response is thought to be of adaptive survival value during periods of deprivation of environmental sources of amino acids for amino acid requiring strains). We have determined that strains which are isogenic except for the stringency gene, do indeed differ in their resistance to oxygen poisoning. The stringent strain, like most wild-type strains, survived 4.2 atm of oxygen with little or no killing for 21 hrs, while the relaxed strain (which does not produce additional ppGpp in response to intracellular dearth of amino acid) showed measureable decrease in viability. The recovery response following removal from hyperoxia was

very different for the two strains. Inhibition of growth was reversed in the stringent strain much more rapidly than in the relaxed strain. Two conclusions regarding the biological role of stringency with respect to survival during the stress of oxygen toxicity are apparent. For a stringent strain, compared to a relaxed strain: (1) more cells would survive and, (2) growth would occur much sooner upon removal from the elevated oxygen state. In direct competition, the relaxed strain would be selected against so that it probably would not survive. The data are shown in Figure 3.

(2) Further evaluation of effects of oxygen on the NAD-niacin pathway (parts 2-b-i through iii):

The proposed research has not been completed. A paper, summerizing progress to date, has been submitted to Biochimica et Biophysica acta. A copy is enclosed as an addendum I. In addition, significant progress has been made in closely correlated research which was not specifically proposed. This research was made possible by the addition of Dr. Foudin to our research group, at no cost to ONR.

Of basic importance is the determination of the extent to which discoveries made with the bacterial sites of oxygen toxicity may apply to cells of higher life forms. To this end, assays of PRPP synthetase activity in erythrocytes, in mouse fibroblast and in Hela cells have been made.

Exposure of whole human blood to hyperbaric oxygen for 30 min. resulted in only a small reduction (about 12%) in PRPP synthetase specific activity (Table 1). Of striking interest, however, is the finding that the PRPP content in the erythrocytes was greatly increased during the period of oxygen exposure. This indicates that biosynthetic

reactions which utilize PRPP are greatly impaired. The poisoning of quinolinate transferase could well account for this, and its activity will be measured.

The data for Hela and mouse fibroblast cells does not reveal any significant impairment of PRPP synthetase during oxygen exposure (Table 2). The PRPP content prior to and following hyperbaric oxygen exposure has not yet been determined in these cells.

It was anticipated that PRPP synthetase specific activity would be significantly reduced in all these cells (Tables 1 and 2) by incubation in hyperoxia. The contrary finding is of interest and may be eventually explained by one of the following: (a) we are in error with respect to the data from either the bacteria or the mammalian cells, (b) the mammalian cells are more resistant due to protective levels of enzymes such as superoxide dismutase, or (c) toxic levels of oxygen radicals were not generated under the conditions of the experiment. We do not feel that (a) is a likely explanation. The resistance of erythrocytes may result from a combination of high concentrations of superoxide dismutase and other protective factors and a low production of oxygen radical species. This would be in keeping with the known function of the red cell as a carrier of oxygen. The resistance of Hela and mouse fibroblasts is more unexpected. The cultures were mature and may actually have been non-proliferating. The actual metabolic rates (including oxygen uptake and subsequent production of radicals) may have been low. Further experiments with young cultures and actual determinations of metabolic rates in hyperoxia are planned. If these data patterns hold, it indicates that poisoning of PRPP synthetase may not be significant to oxygen toxicity in mammals.

The previously mentioned (Table 1) increase in PRPP concentration in erythrocytes after incubation in hyperoxia is most interesting. It possibly is related to our previous findings that: (a) quinolinate transferase (which requires PRPP as a substrate) is poisoned in hyperoxia, and (b) there is impaired synthesis of nucleotides and RNA (which requires PRPP as substrate).

To date, comparisons of the physical characteristics of the enzymes which we have found to be oxygen sensitive, has not revealed any common properties which are definitely associated with the sensitivity. Compilation from the literature of features of the sensitive enzymes is continuing.

(3) In vivo inactivation of Fructose-1,6-diphosphatase by hyperoxia (parts 7-b-i).

A surprising result was obtained when fructose-1,6-diphosphatase specific activity was compared in extracts of Escherichia coli K-12 prior to and following exposure to hyperoxia. Inactivation of the enzyme was minimal (Table 3).

It may be recalled that our previous data had shown that: (a) fructose-1,6-diphosphate (a substrate in the Krebs cycle) was a much poorer protective agent than was fructose-6-phosphate, fructose, or glucose. This was interpreted as evidence that reverse glycolysis was impaired at the enzyme step catalyzed by fructose,1-6-diphosphatase [refer to report #30, December 29, 1977 for a fuller analysis].

When purified fructose-1,6-diphosphate was exposed in vitro to superoxide anion, there was measurable inactivation, while under similar conditions, another control enzyme was not affected. We ten-

tatively concluded that the block in reverse glycolysis in hyperoxia was due to the oxygen sensitivity of fructose-1,6-diphosphatase, but proposed in vivo experiments to seek confirmation.

Results of these experiments, as previously stated (Table 3), necessitated a further evaluation by the technique of measuring protection with various intermediates, which was beyond the experiments proposed in the contract.

Table 4 shows the results of these experiments with K12. As previously determined with E26, glucose was the best protective compound, and fructose-6-phosphate was almost as good. Also, as previously determined, fructose-1,6-diphosphate and pyruvate did not protect as well. However, we went further, after these confirmatory experiments to measure whether the supplemented carbohydrates were actually utilized by the bacteria. The test of utilization was to compare the total growth obtained in the presence and in the absence of the added carbohydrate. A new fact was uncovered here. Among the carbohydrates tested (Table 4) only fructose-1,6-diphosphate failed to increase the total yield of cells. This clearly showed that the failure of fructose-1,6-diphosphate to protect was due to incapability of the cells to transport it, and was not due to poisoning of fructose-1,6-diphosphatase which agrees with the data of Table 3.

To further establish this point, glycerol was tested as a substrate (Table 4). It is taken up by the cells and gives protection comparable to fructose-6-phosphate. Glycerol enters the EMP as glyceric acid-3-phosphate (prior to PEP). Together, these data implicate oxygen impairment of phosphoenolpyruvate carboxykinase and phosphoenol pyruvate

synthetase as the cause of inhibition in reverse glycolysis.

Fructose-1,6-diphosphatase, which was previously suspect, is not the site in vivo, and our preliminary conclusions were in error because fructose-1,6-diphosphate does not enter the cell, presumably because of its highly polar nature due to the two phosphates.

Tests of the effect of hyperoxia on phosphoenolpyruvate carboxykinase and phosphoenolpyruvate synthetase activity in cell extracts, are proposed.

(4) Evaluation of the specific enzymatic site of oxygen poisoning of branched chain amino acids. [This research was done to complete objectives of the proposal for the period April 1, 1977 through March 31, 1978.] The research is described in the attached copy of a manuscript, addendum II.

I.C. Personnel associated with the project and funding sources:

Considerable salary support for research on the objectives of the current ONR contract has been provided by University sources.

(a) Olen R. Brown, Ph.D. Principal Investigator, salary paid by University of Missouri.

(b) Laurie Foudin, Ph.D., Post-doctoral trainee, salary paid by NIH Training Grant for the Dalton Research Center, Sept, 1, 1978 - August 31, 1979.

(c) Patti Gilliland, M.S., Research Specialist, salary paid by ONR contract, Sept. 1, 1978 - March 31, 1979.

(d) Frederick Yein, M.S. Research Specialist, salary paid by ONR contract and the Dalton Research Center, April 1, 1978 - July 1978.

(e) Richard Siether, B.S., Graduate student, stipend paid by Dalton Research Center and Graduate School.

(f) Doris, Song, M.S., temporary research Specialist, salary paid by ONR.

I.d. Publications

(In Press or published since renewal request of 1978).

(a) Brown, O. R., Yein, F., and Boehme, D. Bacterial Sites of Oxygen Toxicity Potentially Common to Red Cells and Erythropoiesis. The Red Cell, Proceedings 4th International Conference on Red Cell Metabolism and Function, Ed. by G.J. Brewer, Alan R. Liss, Pub., pp. 701-714, (1978).

(b) Brown, O. R., Yein, F. Sensitivity to and Site of oxygen Poisoning in Escherichia coli. Fifth International Symposium on Intestinal Microecology, Ed. by D. Hentges and T.D. Lucky. In the Press: American Journal of Clinical Nutrition 32 (1979).

(c) Brown, O. R. and Yein, F. Dihydroxyacid Dehydratase: The Site of Hyperbaric Oxygen Poisoning in Branched-chain Amino Acid Biosynthesis. Biochem. Biophys. Res. Comm., 85:1219-1224 (1978).

(d) Brown, O. R., Boehme, D., and Yein, F. Fructose-1,6-diphosphatase: A Cellular Site of Hyperbaric Oxygen Toxicity. (In the Press, Microbios., 1978).

(e) Brown, O. R., Yein, F., and Boehme, D. Oxygen Poisoning of NAD and Phosphoribosylphosphate Biosynthesis: Proposed Sites of Cellular Oxygen Toxicity. (Submitted to Biochimica Et Biophysica Acta).

(f) Brown, O. R. Specific Enzymatic Sites and Cellular Mechanisms of Oxygen Toxicity, (In the Press: Proceedings of 2nd International Conference on Biochemical and Clinical Aspects of Oxygen. Ed. by W.S. Caughey).

Table 1. Effect of Hyperbaric Oxygen on PRPP Synthetase Activity in Erythrocytes Following Treatment of Whole Blood

Treatment prior to assay*	Enzyme activity** (nmoles PRPP/mg/hr)	Relative PRPP content (dpm/assay)***
Control (no treatment)	53.58±5.04	1302±662
HPN (4.8 atm, 30 min)	52.78±4.55	2670±322****
HPO (4.2 atm, 30 min)	47.48±5.40****	5889±1230****

*Whole blood was incubated at 37°C under the specified conditions. Following treatment, erythrocyte hemolysates were prepared and assayed for enzyme activity. The relative PRPP content are the values for extracts incubated without substrates.

**Averages ± 1 S.D. For 12 determinations for 3 experiments.

***Averages ± 1 S.D. for 9 determinations from 3 experiments.

****$P \geq 0.01$, student's t-test.

Table 2. Effect of Hyperbaric Oxygen on PRPP Synthetase Activity in Mammalian Cells

Treatment prior to assay*	Enzyme Activity (μmoles PRPP/mg/hr)	
	Hela	Mouse Fibroblasts
Control	1.94 (2)**	0.403±0.051 (4)
HPN (2.8 atm, 60 min)	2.71 (2)	0.567±0.041 (4)***
HPO (2.2 atm, 60 min)	2.09 (2)	0.469±0.104 (4)

*Cells were incubated at 37°C under the specified conditions. Following treatment, cell-free extracts were prepared and assayed. With Hela Cells (monolayer), the control was prepared directly without further incubation; with fibroblasts, cell-suspensions from monolayers were prepared prior to treatment, and the control suspension was incubated in air for 60 min.

**Number in parentheses indicates number of determinations; with fibroblasts, two concentrations of extract were used.

***$P \geq 0.01$, student's t-test.

Table 3. Effect of High Pressure Oxygen Exposure on Fructose-1,6-Diphosphatase in Escherichia coli K12[1].

Time of exposure (min)	Specific Activity (U/mg)[2]	
	Air	HPO
10	0.172±.022 (12)	0.234±.035 (12)[3]
60	0.172±.032 (24)	0.145±.030 (23)[4]

[1]Cells were exposed to 4.2 atm partial pressure of oxygen (HPO) for the indicated time at 37°C during exponential growth. The enzyme activities were measured in cell-free extracts by the phosphate liberation method of Pontremoli (Methods Enzymology 9:625-631) with inorganic phosphate determined by a modification of the method of Fiske and Subbarow (J.B.C. 66:375-380).

[2]A unit of fructose 1,6-diphosphatase activity liberates one micromole of inorganic phosphate in one minute at 37°C. The numbers in parentheses represent the number of total assays.

[3]Significantly higher than air control at $p \leq 0.005$.

[4]Significantly lower than air control at $p \leq 0.001$, using Student's t-test.

Table 4. Effect of specific carbohydrates on Growth in hyperoxia of Escherichia coli K12.

Supplemented Carbohydrate[1]	Gen. Time (min)		Percentage Protection	#gen/hr	Normalized Ratio[2]
	Air	4.2 atm O_2		4.2 atm O_2	
Glucose[3]	38.2±2.34	50.4±6.31	76.6±8.71	1.21±1.44	1.00
Fructose-6-phosphate[3]	28.9±1.62	53.2±3.42	55.1±4.5	1.15±0.68	0.72
Fructose-1,6-diphosphate[4]	37.7±2.72	250.8±46.7	15.6±4.44	0.248±0.52	0.20
Glycerol[3]	34.8±1.09	64.6±3.38	54.1±4.6	0.936±.05	0.71
Pyruvate[5]	36.7±1.16	99.4±1.31	37.0±1.2	0.608±.008	0.49

[1]Medium contains 20 amino acids.

[2]The quotient obtained by dividing the ratio of the air to hyperbaric oxygen generation times by the value of this ratio with glucose present. This ratio compensates for the differences in growth rates in the various media and is normalized with respect to the protection with glucose present. The value of normalized rations decrease as protection by the supplimented carbohydrate decreases, relative to protection by glucose.

[3]Enters the EMP pathway prior to PEP.

[4]Determined by experiments to not enter the cell.

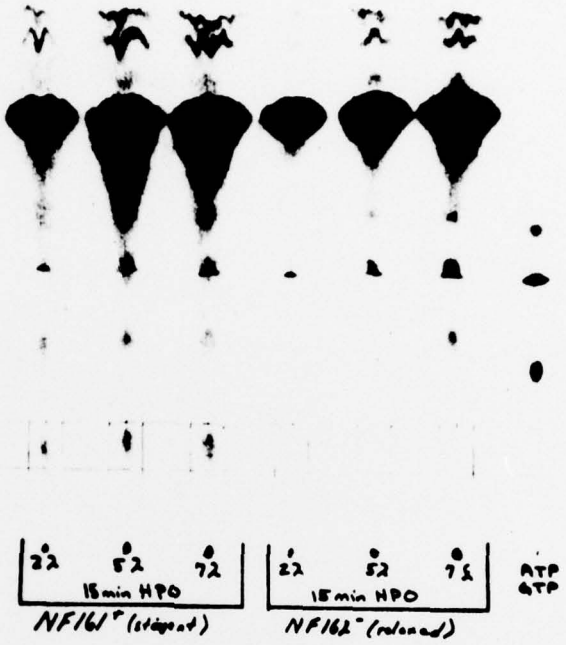
[5]Enters the EMP pathway after PEP.

Figure Legends

Figure 1. Photograph of thin-layer chromatograms of ppGpp produced by stringent and relaxed *E. coli* strains following exposure to toxic levels of oxygen.

Figure 2. Comparative effects of hyperoxia on stringent and relaxed strains. More cell death (during hyperbaric oxygen exposure) and slower recovery of growth (upon removal from hyperoxia) occurred with the relaxed compared to the stringent strain. The relaxed strain does not produce ppGpp and "unbalance" metabolism continues for a period of time in hyperoxia.

FIGURE 1



The spots appearing in the marked squares on the bottom third of the photograph are ppGpp. They are absent in the relaxed strain but are present in the stringent strain following 15 minutes in 4.2 atm of oxygen.

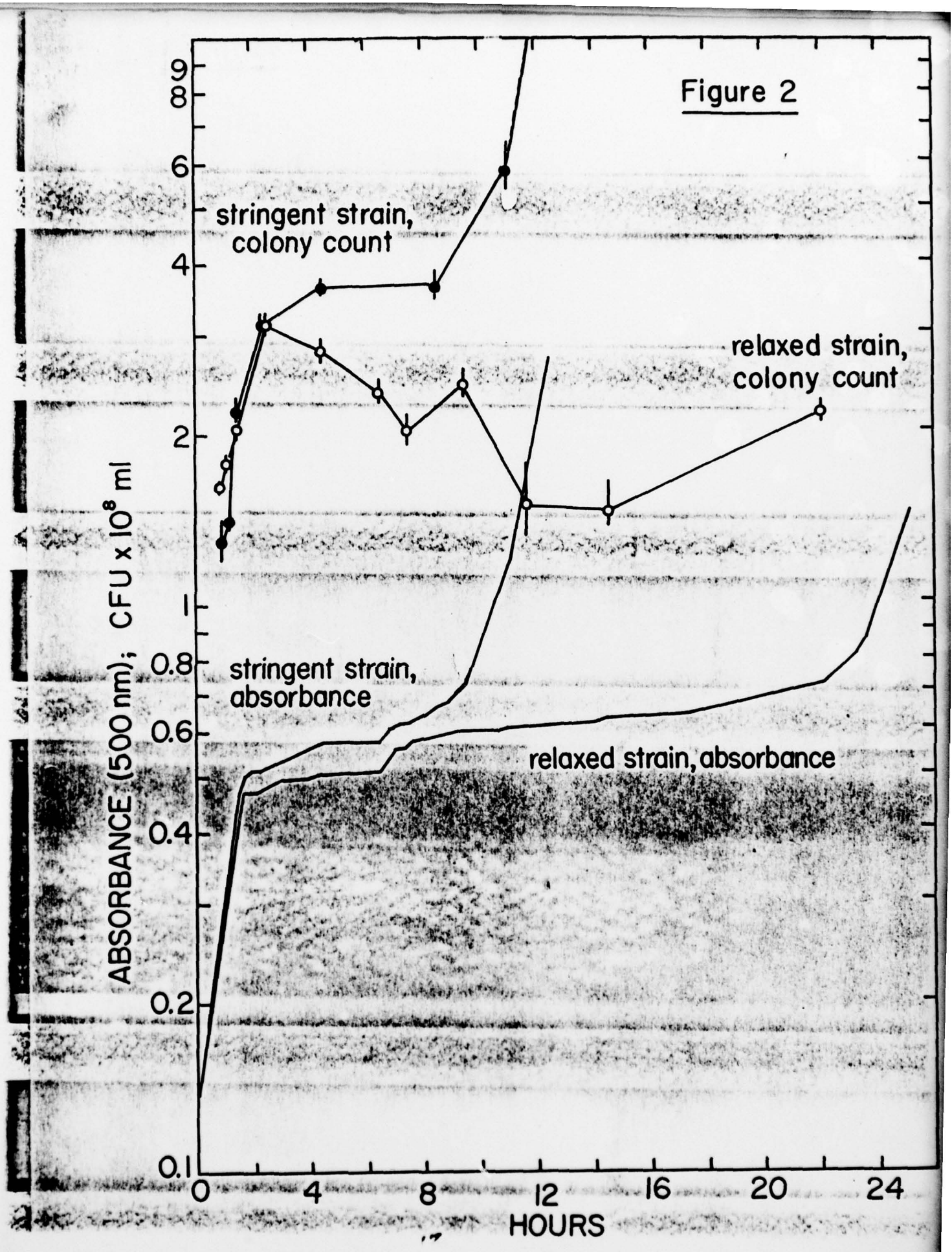


Figure 2
stringent strain, colony count
relaxed strain, colony count
stringent strain, absorbance
relaxed strain, absorbance
ABSORBANCE (500 nm); CFU x 10⁸ ml
HOURS
0.1
0.2
0.4
0.6
0.8
1
2
4
6
8
9
0
4
8
12
16
20
24